WO9419471

Publication Title:

METHOD AND SYSTEM FOR ENHANCED PRODUCTION OF COMMERCIALLY IMPORTANT EXOPROTEINS IN GRAM-POSITIVE BACTERIA

Abstract:

Abstract of WO9419471

The invention provides a method and expression system for enhancing secretion of hyperproduced homologous and heterologous exoproteins in gram-positive bacteria such as Bacillus sp. The method and system comprise overproduction of PrsA protein in a gram-positive bacterial host capable of also overproducing at least one exoprotein of interest. Use of the method and system of the invention results in greatly enhanced secretion of the synthesized exoproteins into the growth medium. Once in the growth medium these secreted exoproteins can be recovered and purified in a straightforward manner. Data supplied from the esp@cenet database - Worldwide

---

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 15/67, 15/74, 15/75 — A1

(11) International Publication Number: WO 94/19471

(43) International Publication Date: 1 September 1994 (01.09.94)

(21) International Application Number: PCT/FI94/00072

(22) International Filing Date: 25 February 1994 (25.02.94)

(30) Priority Data:
08/024,154 26 February 1993 (26.02.93) US

(60) Parent Application or Grant
(63) Related by Continuation
US 08/024,154 (CIP)
Filed on 26 February 1993 (26.02.93)

(71) Applicant *(for all designated States except US)*: THE FINNISH NATIONAL PUBLIC HEALTH INSTITUTE [FI/FI]; Mannerheimintie 166, FIN-00300 Helsinki (FI).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: KONTINEN, Vesa [FI/FI]; Kuhnurintie 2, FIN-53650 Lappeenranta (FI). SARVAS, Matti [FI/FI]; Pirttipolku 18 A, FIN-00630 Helsinki (FI).

(74) Agent: OY KOLSTER AB; Iso Roobertinkatu 23, P.O. Box 148, FIN-00121 Helsinki (FI).

(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, GE, HU, JP, KP, KR, KZ, LK, LU, LV, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: METHOD AND SYSTEM FOR ENHANCED PRODUCTION OF COMMERCIALLY IMPORTANT EXOPROTEINS IN GRAM-POSITIVE BACTERIA

(57) Abstract

The invention provides a method and expression system for enhancing secretion of hyperproduced homologous and heterologous exoproteins in gram-positive bacteria such as *Bacillus sp.* The method and system comprise overproduction of PrsA protein in a gram-positive bacterial host capable of also overproducing at least one exoprotein of interest. Use of the method and system of the invention results in greatly enhanced secretion of the synthesized exoproteins into the growth medium. Once in the growth medium these secreted exoproteins can be recovered and purified in a straightforward manner.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgystan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo			SE	Sweden
CH	Switzerland	KR	Republic of Korea	SI	Slovenia
CI	Côte d'Ivoire	KZ	Kazakhstan	SK	Slovakia
CM	Cameroon	LI	Liechtenstein	SN	Senegal
CN	China	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
CZ	Czech Republic	LV	Latvia	TJ	Tajikistan
DE	Germany	MC	Monaco	TT	Trinidad and Tobago
DK	Denmark	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	US	United States of America
FI	Finland	ML	Mali	UZ	Uzbekistan
FR	France	MN	Mongolia	VN	Viet Nam
GA	Gabon				

Method and System for Enhanced Production of Commercially Important Exoproteins in Gram-Positive Bacteria

Field of the Invention

This invention relates to a method and expression system for enhanced production of industrially and medically important exoproteins in gram-positive bacteria, especially species of the genus *Bacillus*.

Background

In gram-positive bacteria secreted proteins are exported across a cell membrane and a cell wall, and then are subsequently released into the external medium. On the other hand, gram-negative bacteria are surrounded by two cell (or surface) membranes; they have no cell wall. In gram-negative bacteria, most exported proteins are not released from the cell but stay in the inter-membrane periplasmic space and in the outer membrane.

Two types of components of the secretion machinery have been identified in *E. coli*: soluble cytoplasmic proteins and membrane associated proteins (*see* for review, Wickner *et al.*, (1991) *Annu. Rev. Biochem.*, 60:101-124). Soluble cytoplasmic proteins, including SecB and heat shock proteins, all prevent the folding of precursors of secreted proteins into a conformation incompatible with secretion. The set of membrane-associated proteins includes the peripheral membrane protein SecA, integral membrane proteins SecY, SecE, SecD, SecF and the signal peptidases Lep and Lsp (reviewed in Hayashi, S. and Wu, H.C. (1990) *J. Bioenerg. Biomembr.*, 22:451-471; Dalbey, R.E. (1991) *Mol. Microbiol.*, 5:2855-2860). These membrane-associated proteins are involved in binding of the precursor and in its translocation across the cytoplasmic membrane, followed by cleavage of the signal peptide and release of the protein.

Knowledge on the secretion machinery of gram-positive bacteria is more limited. The available data on *B. subtilis*, the genetically and physiologically well characterized model organism of the genus, suggest an

overall similarity with that of *E. coli*, but also differences in the structure and specificity of individual components, possibly reflecting demands set by the very different composition and architecture of the respective cell envelopes.

Gram-positive bacteria such as *B. subtilis, B. amyloliquefaciens, B. licheniformis* have a very high capacity for secreting proteins, and indeed, many bacillar extracellular enzymes are utilized industrially. Since secreted proteins in gram-positive bacteria are so important commercially, and since the gram-positive secreted proteins traverse through a cell envelope with a very different structure from that of *E. coli*, the molecular mechanisms of protein secretion in gram-positive bacteria is of considerable academic and industrial importance.

In this regard we recently discovered a novel component, the PrsA protein, of the secretion machinery of *B. subtilis* (Kontinen, V.P. and Sarvas, M., (1988) *J. Gen. Microbiol.*, 134:2333-2344; Kontinen, V.P., *et al.*, (1991) *Mol. Microbiol.* 5:1273-1283). The *prsA* gene, which encodes the PrsA protein, was initially defined by nonlethal mutations that decreased the secretion of several exoproteins (Kontinen, V.P. and Sarvas, M., (1988) *J. Gen. Microbiol.*, 134:2333-2344). Based on the DNA sequence of the cloned *prsA* gene and our subsequent work with this gene and protein, we assert that *prsA* encodes a protein (PrsA) that acts as a chaperone, and is translocated across the cytoplasmic membrane (for the initial work, *see* Kontinen, V.P., *et al.*, (1991) *Mol. Microbiol.* 5:1273-1283). The PrsA protein has been found to possess a limited amount of sequence homology (about 30%) with the PrtM protein of *Lactococcus lactis*, a protein proposed to assist the maturation of an exported serine protease (Haandrikman, A.J., *et al*, (1989) *J. Bacteriol.*, 171:2789-2794; Vos, P., *et al.*, (1989) *J. Bacteriol.*, 171:2795-2802). A similar function has not been associated with other known proteins of the secretion machinery of bacteria, suggesting that PrsA protein is a novel type of component in the pathway of protein secretion facilitating the release and probably folding of secreted proteins after their translocation across the cytoplasmic membrane in gram-positive bacteria.

It is advantageous to produce proteins of interest in bacteria in secreted form, since exported proteins usually maintain their native conformation, in contrast to intracellular production which, in many cases, results in aggregation of the produced protein. Another advantage of producing industrially and medically important proteins in bacteria in secreted form is that secretion facilitates purification of the protein product. Additionally, unlike *E. coli*, gram-positive bacteria such as *Bacillus* sp. do not contain toxic compounds like lipopolysaccharide, making them especially appropriate hosts for production of medical and pharmaceutical proteins.

Increased yield of secreted proteins would be of great significance for improving the gram-positive bacillar strains used in the industrial production of a number of exoenzymes, such as alpha-amylases, proteases and lipases. The strategy thus far has been to overexpress the appropriate gene. There are known and readily available methods for doing this, such as increasing gene expression by using multicopy plasmids or enhancing the activity of the gene by modifying its regulatory elements, *e.g.*, by using strong promoters, or multiple promoters. Dramatic increases of the synthesis of exoproteins have been achieved this way, up to a level at which increasing the synthesis is of no further benefit because of bottlenecks in the secretion machinery. It would be desirable to increase the capacity of secretion in parallel with increased synthesis. However, to date this has not been possible.

It is an object of the present invention to alleviate the bottleneck of the secretion mechanism in gram-positive bacteria, and to provide a method and a system whereby the levels of proteins normally secreted from gram-positive bacteria such as *Bacillus* can be enhanced when the expression of a given homologous or heterologous protein of interest has been elevated over the amount normally produced in unmodified or wild type organisms.

It is a further object of the present invention to describe bacterial hosts and plasmids which can be used to enhance the production of a variety of commercially important exoproteins.

Summary

The invention provides a method and expression system for enhancing the levels of homologous or heterologous protein(s) normally secreted from gram-positive bacteria (such as *Bacillus sp.*) when expression of the homologous or heterologous protein(s) has been elevated over unmodified or wild type amounts produced by unmodified or wild type organisms.

The method and system of our invention comprise overproduction of PrsA protein, or a functional homologue thereof, in a gram-positive bacterial host capable of also overproducing at least one homologous or heterologous exoprotein of interest. According to the teaching of the invention, overproduction means an amount greater than wild-type, *i.e.*, more than the amount of the protein (PrsA or a functional homologue thereof, or exoprotein of interest) normally produced by wild type bacteria. Also according to the invention, overproduction is accomplished by standard means known to the art, *e.g.*, use of multicopy plasmids, multiple copies of the genes encoding PrsA, or a functional homologue(s) thereof, and/or the exoprotein of interest, in the chromosome of the host, combined with altering the regulatory elements to increase expression, *e.g.*, use of strong promoter(s), use of multiple promoters, use of enhancers, and so forth. Use of the method and system of the invention results in greatly enhanced secretion, *e.g.*, as much as five to ten fold over controls, of synthesized exoproteins into the growth medium. Once in the growth medium these secreted exoproteins can be recovered and purified in a straightforward manner.

The expression system of the invention comprises a host gram-positive bacteria, *e.g.*, species of *Bacillus*, capable of expressing greater than wild-type amounts of PrsA protein, or a functional homologue thereof, and greater than wild-type amounts of an exoprotein of interest, *e.g.*, alpha amylase, subtilisin, pneumolysin, lipases, or other exoproteases of commercial interest. The method of the invention comprises using this expression system to enhance production of commercially important

exoproteins in gram-positive bacteria. According to the method, at least one exoprotein of interest is overexpressed in a host gram-positive bacteria which also overexpresses (*i.e.*, expresses greater than the amounts produced by wild type bacteria) PrsA protein, or a functional homologue thereof. According to the teaching of the invention, a functional homologue of PrsA protein is a protein which when overexpressed is capable of enhancing the secretory capability of a gram-positive bacteria with respect to secretion of an exoprotein of interest. Also according to the teaching of the invention, a functional homologue of PrsA can be identified by several means including sequence homology to *prsA* or PrsA, immunological reaction with anti-PrsA antibod(ies) of high titer, and/or functionally, *i.e.*, as a protein which when overexpressed, is capable of enhancing the secretory capability of a gram-positive bacteria with respect to secretion of an exoprotein of interest.

A preferred means for transforming host gram-positive bacteria, such as *species of Bacilli*, so they produce greater than wild-type amounts of PrsA protein is to transform the host with plasmid pKTH277 which carries the *prsA* gene from *Bacillus subtilis*. Comparable plasmids can be constructed to carry genes which encode functional homologues of the PrsA protein. These plasmids can be used to transform host gram-positive bacteria so they overproduce the functional homologues of PrsA. Once engineered to overproduce PrsA homologues (which can also be referred to as PrsA-like proteins), these host gram-positive strains can be used, according to the teaching of the invention, for enhanced secretion of hyperproduced exoproteins of interest.

The present invention also discloses, and includes, methods and constructs related to our discovery that secretion in gram-positive bacteria can be enhanced by increasing the amount of cellular PrsA protein, or functional homologue(s) thereof, in gram-positive hosts that express greater than wild-type amounts of exoproteins of interest.

In one aspect our invention includes an expression system for enhancing secretion of exoproteins in gram-positive bacteria comprising a gram-positive bacteria capable of expressing greater than wild-type amounts

of PrsA protein, or functional homologue(s) thereof, and capable of expressing greater than wild-type amounts of at least one exoprotein of interest.

In another aspect our invention includes a gram-positive bacteria able to express greater than wild-type amounts of at least one exoprotein of interest further comprising pKTH277.

In yet another aspect, our invention includes a gram-positive bacteria able to express greater than wild-type amounts of at least one exoprotein of interest and further comprising at least one of the following: at least two copies of the *prsA* gene from *Bacillus subtilis*, or a functional homologue thereof; the *prsA* gene from *Bacillus subtilis*, or a functional homologue thereof, operatively linked to strong regulatory sequences which result in overexpression of said *prsA* gene, or functional homologue thereof.

Our invention also includes a DNA construct comprising the *prsA* gene from *Bacillus subtilis*, or a functional homologue thereof, under the control of expression signals which cause overexpression of said *prsA* gene, or functional homologue thereof, plus a vector further comprising the *prsA* gene from *Bacillus subtilis*, or a functional homologue thereof, under the control of expression signals which cause overexpression of said *prsA* gene, or functional homologue thereof.

In yet another aspect our invention includes a method for enhancing secretion of an exoprotein of interest in a gram-positive bacteria comprising expressing greater than wild type amounts of PrsA protein from *Bacillus subtilis*, or a functional homologue thereof in the gram-positive bacteria, wherein the gram-positive bacteria is also capable of expressing greater than wild type amounts of the exoprotein.

Still further the invention includes a method for creating an improved non-*Bacillus subtilis* gram-positive host organism useful for enhanced secretion of an exoprotein of interest that is overexpressed in the host organism, the method comprising (a) identifying a gene from the host organism that encodes a functional homologue of PrsA protein from *Bacillus subtilis*, and (b) enhancing the expression of the gene identified in step (a) by

at least one of the following: introducing into the host organism at least one additional copy of the gene; introducing into the host organism the gene operatively linked to expression sequences which result in overexpression of the gene.

The invention also includes a method for identifying a gene which encodes a functional homologue of PrsA from *Bacillus subtilis*, the method comprising identifying, by means of Southern blotting, DNA which hybridizes with DNA probe(s) from the *prsA* gene from *Bacillus subtilis*, and demonstrating that the gene encodes a protein which when overexpressed, is capable of enhancing the secretory capability of a gram-positive bacteria with respect to secretion of an exoprotein of interest.

Still further, the invention includes a method for identifiying a gene which encodes a functional homologue of PrsA from *Bacillus subtilis*, this method comprising identifying protein that reacts with anti-PrsA antibod(ies) of high titer, and demonstrating that when the protein is present in greater than wild-type amounts in a gram-positive bacteria, the protein is capable of enhancing the secretory capability of the gram-positive bacteria with respect to secretion of an exoprotein of interest.

These and other features, aspects and advantages of the invention will become better understood with reference to the following description, examples, methods and materials, and appended claims.

Description

When the expression level (synthesis) of an exported protein is high in gram-positive bacteria such as *Bacillus sp.*, the capacity of the secretion apparatus is a limiting factor in protein secretion and production of these proteins in secreted form. Our invention provides a system and method for overcoming this limitation or bottleneck.

Our invention is based on our initial surprising discovery that secretion in gram-positive bacteria such as species of *Bacillus* can be enhanced by increasing the amount of only one component of the bacillar export machinery, *i.e.*, the amount of cellular PrsA protein, or functional

homologues thereof, in gram-positive bacterial hosts that express greater than wild-type amounts of exoproteins of interest. The method and system of the invention are useful regardless of how the proteins of interest are overproduced in the gram-positive bacterial host. Thus the method and system can be used to improve a variety of overproducing commercial strains now used in industrial applications.

The method and system of the invention can be used with any gram-positive bacteria. Bacteria of the genus *Bacillus* are preferred. Especially preferred are *Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus brevis, Bacillus circulans, Bacillus coagulans, Bacillus lautus, Bacillus lentus, Bacillus licheniformis, Bacillus stearothermophilus, Bacillus subtilis,* and *Bacillus thuringiensis.*

The method and system of the invention can also be used with any desired exoprotein of interest. Examples of exoproteins of interest that may be produced according to the method and system of the invention are listed below, where the exemplary exoproteins are presented by general categories.

Antigenic proteins of microbes and protozoa: Capsule, outer membrane and fimbria proteins from any gram negative bacteria, but especially those from: *Bacteroides fragilis*, Fusobacterium spp., *Bordetella pertussis, Haemophilus influenzae, Yersinia entercolitica, Yersinia pestis, Branhamellla catarrhalis, Escherichia coli, Klebsiella pneumonia, Vibrio cholerae, Proteus mirabilis, Pseudomonas aeruginosa, Serratia marcescens, Legionella pneumophila, Neisseria gonorrhoeae, Neisseria meningitidis, Salmonella typhimurium, Salmonella typhi, Salmonella pararyphi B. Mycobacterium tyberculosis, Chlamydia trachomatis*, and Shigella spp.

Protein toxins or secreted proteins from any bacteria, but especially those from: *Staphylococcus aureus, Pseudomonas aeruginosa, Clostridium spp., Escherichia coli, Yersinia pestis, Vibrio cholerae, Bordetella pertussis*, M-Protein of the *Streptococcus pyogenes* bacterium, Excreted enzymes of Stretococcus mutans.

Surface proteins of any microorganism, but especially those from the following microorganisms (in all phases of development): Plasmodium spp., Toxoplasma spp., Leishmania spp., Schistosoma spp., Trypanosama spp. Adhesion protein of *Streptococcus* sp., and adhesion protein of *Staphylococcus aureus.*

Antigen proteins or viruses: HA and NA proteins of myxoviruses (influenza A H1-H12, influenza B, influenza C): HN and F proteins of paramyxoviruses (parainfluenze 1-4, Newcastle disease virus, Measles virus, Respiratory syncytial virus, Parotitis virus, Distemper virus): G protein of Rabies virus; E1 and E2 proteins of alfaviruses (Chikungunya, Western, Easter, Venezuelan equine encephalitis virus, O'nyong-nyong virus, Semliki Forest virsu, Sindbis virus); V1 and V3 proteins of flavin viruses (Denque 1-4, Japanese encephalitis virus, Mite encephalitis viruses, Murray Valley encephalitis virus, Kyasanur Forest disease virus, Looping ill virus, Omsk hemorrhagic fever virus); surface proteins of German measles virus; surface proteins of Hog Cholera virus; surface proteins of Equine arthritisvirus; G1 and G2 proteins of Bunya viruses (Rift Valley fever virus, Crimean hemorrahagic fever virus, California encephalitis virus, Phlebotomus fever virus); G1 and G2 proteins of arena viruses (Lassa fever virus, Lymphocytic chorion meningitis virus); proteins V1-V4 of picorna viruses (polio 1-3, Coxsackie A viruses 1-24, Coxsackie B viruses 1-6, ECHO viruses 1-8, 11-34, hepatitis A virus, hepatitis B virus, hepatitis C virus, Human rhino viruses 1-113); surface proteins of rota viruses; surface proteins of herpes viruses (HSV 1, 2, Cytomegalo virus, Epstein-Barr virus, Equine abortion virus); VP1-VP3 proteins of papova viruses (BK virus, Human wart virus); p proteins of parvo viruses (mink enteritis virus, Bovine parvo virus, Feline parvo virus, Procine parvo virus); structure proteins of Human hepatitis B virus; surface proteins of Ebola and Marburg viruses; and Hexone, pentone and fiber proteins of adeno viruses, (Human adeno viruses 1-33).

Industrial enzymes: With regard to industrially important enzymes, such enzymes may be amylolytic, lipolytic and proteolytic enzymes, carbohydrases, transferases, isomerases, peroxidases, oxidoreductases,

oxidases *etc.* More specifically, the enzyme of interest may be a protease, a lipase, a cutinase, an amylase, a galactosidase, a pullulanase, a cellulase, a glucose isomerase, a protein disuphide isomerase, a CGT'ase (cyclodextrin gluconotransferase), a phytase, a glucose oxidase, a glucosyl transferase, laccase, bilirubin oxidase, or a xylanase. Examples include, but are not limited to: alpha-amylase, amino acid acylase, amyloglucosidase, bromelain, phisine, beta-galactosidease, beta-gulcanase, glucose-ismorase, glucoseoxidase, hemicellulase, invertase, catalase, collagenase, xsylanase, lactase, lipase, naringinase, pancreatin, papain, pectinase, penicillinamidase, pepsin, protease, pullulanase, isoamylase, rennin, ribonuclease, cellulase, streptokinase and trypsin.

Exoproteins of medical interest can also be produced. Such proteins include diagnostic antigens, proteins that can be used as vaccines, and pharmaceuticals.

According to the teaching of the invention, the exoproteins of interest need not be native exoproteins, but instead can be novel proteins that have been designed and created to be exoproteins using genetic engineering techniques. For example, a normally non-secreted protein from one species (or an engineered non-native protein) can be engineered to be an "exo" protein by adding a signal sequence to the sequence encoding the structural protein. This engineered exoprotein can be expressed in a gram-positive bacteria such as a species of *Bacillus*, which overexpresses PrsA protein, or a functional homologue thereof. In this way the method of the invention can be used to enhance secretion of these non-native or engineered proteins of interest.

Turning now to aspects of our invention, to illustrate one embodiment of our invention we show the effect overexpression of the *prsA* gene from *Bacillus subtilis* has on the secretion of the two important industrial exoenzymes of *Bacilli*, alpha-amylase and subtilisin. For these studies, a 5.3 kB insert, containing the entire *prsA* gene from *Bacillus subtilis*, was cloned into a low copy number shuttle plasmid (pKTH277), which was then used to introduce additional copies of *prsA* into *Bacillus subtilis*. (The DNA and

deduced amino acid sequences of the *prsA* gene from *Bacillus subtilis* appear in the EMBL/GenBank/DDBJ Nucleotide Sequence Data Libraries under the accession number X57271.) (pKTH277 was obtained by ligating the 5.3 kB *Eco*RI-*Bam*HI fragment from pKTH268 with low copy number shuttle plasmid pHP13 linearized by digestion with respective restriction enzymes and transforming into the *E. coli* strain TG1. The sizes of pKTH268 and pKTH277 are 8.5 kB and 10.2 kB, respectively. *See* also Kontinen, *et al.*, (1991) *Mol. Microbiol.*, 5:1273-1283, which is incorporated by reference herein). The presence of pKTH277 in *B. subtilis* increased the amount of the protein corresponding to the PrsA protein by approximately 10-fold over the wild type. When the genes for different secreted proteins are expressed in strains of *Bacillus* containing these increased levels of PrsA protein, the level of protein secreted into the culture medium is increased substantially. For example, the secretion of alpha-amylase of *Bacillus amyloliquefaciens* was found to be increased by 2.5-fold in this system, the secreted level of the thermoresistant alpha-amylase of *Bacillus licheniformis* was elevated by six-fold, and subtilisin (alkaline protease) from *Bacillus licheniformis* was secreted at two times the control level.

In these studies the exoenzymes were overexpressed in host strains in amounts likely to saturate the secretion machinery, either by placing the gene which encoded the exoprotein on a multicopy plasmid or inserting it in the chromosome of the host. (In these studies, all multicopy plasmids coding for the exoenzymes were derivatives of pUB 110, which belongs to a different incompatibility group than the shuttle plasmid pKTH277, allowing their replication in the same host cell. The stability of these plasmids was further increased in most cases by using a *recE4* host strain, which prevents efficient recombination between homologous sequences (Dubnau et al., 1973; Keggins et al., 1978).

The first exoenzyme studied to illustrate this aspect of our invention was alpha-amylase of *B. amyloliquefaciens* (AmyE) encoded by pKTH10 (Palva, I. (1982a) *Gene*, 19:81-87; Palva, I., *et al.*, (1982b) *Proc. Natl. Acad. Sci. USA*, 79:5582-5586). We found that in the wild-type strain

hyperproducing this alpha amylase, the presence of pKTH277 indeed enhanced the secretion of alpha-amylase throughout the stationary phase of growth, about 2.5-3 fold over the level of the control strain which did not overexpressing PrsA. The highest concentration of alpha amylase in the culture supernatant (about 3400 micrograms/ml) was found after the growth of 24 hours. In the absence of pKTH277, the strain secreted only 1200 micrograms/ml. Qualitatively similar results were obtained when alpha-amylase was expressed from one copy of the *amyE* gene, which was inserted in the chromosome and transcribed at high level due to modified regulation.

The second exoprotein we tested was the thermoresistant alpha-amylase of *B. licheniformis* (AmyL), the major liquefying alpha-amylase of industrial importance (Ortlepp et al., 1983; Diderichsen, B., *et al.,* (1991) *Res. Microbiol.,* 142,.793-796). Secretion of this enzyme in *B. subtilis* at amounts comparable with those of the alpha amylase of *B. amyloliquefaciens* was achieved by expressing the appropriate gene from the secretion vector based on the promoter and signal sequence of the gene of the latter enzyme (Palva, I. (1982) *Gene,* 19:81-87; Palva, I., *et al.,* (1982) *Proc. Natl. Acad. Sci. USA,* 79:5582-5586); Sibakov, M. (1986) *Eur. J. Biochem., 1sS,* 577-581). Introduction of pKTH277 into one such strain (to result in IH6760) increased the amount of alpha-amylase in the culture medium about six fold, with the same difference seen from late exponential stage to cultures of 45 hours.

Alkaline protease, subtilisin, is a different type of exoprotein, whose precursor contains in addition to the signal sequence a further extension, the prosequence (Wells, *et al.,* (1983) *Nucleic Acids Res.,* 11:7911-7925; Wong, S.-L., *et al.,* (1984) *Proc. Natl. Acad. Sci. USA,* 81:1884-11188). The effect of an increased amount of PrsA on this secretion was studied by comparing two strains, one with increased level of PrsA (IH6789), another one with the wild type level. Both secreted the heterologous subtilisin of *B. licheniformis* (SubC, which is used as a laundry powder and is an important industrial product) coded by the multicopy plasmid pMJ57 (Hastrup, S. and Jacobs, M.F. (1990) In Zukowski, M.M., *et*

al., (eds.), *Lethal phenotype conferred by xylose-induced overproduction of apr-lacZ fusion protein*, vol. 3. Academic Press, Inc., San Diego, California, pp. 33-41). In this plasmid the *subC* gene is under the control of a xylose inducible promoter. Comparison of the secretion of subtilisin from the two strains, when fully induced, showed that its amount in the culture supernatant of IH6789 (increased amount of PrsA) was about twofold higher than that of the control IH6788 at all time points assayed.

We also studied the effect of pKTH277 on the natural low level secretion of endogenous exoenzymes in a strain devoid of any plasmid causing hypersecretion. The amount of secreted alpha amylase and total proteases in the late exponential phase of growth or in overnight cultures was the same in strains carrying pKTH277 or the cloning vector pHP13. Based on these results it appears that the increased amount of PrsA protein enhances secretion of hyperproduced exoenzymes only.

In order to confirm the role of PrsA in the enhancement caused by the 5.3 kb fragment in the above plasmids, we inactivated the *prsA* gene in the plasmid pKTH277 by insertions in the *Eco*RV site of its *prsA* gene (at the nucleotide 382). In pKTH3261 the insert was a 560 bp fragment of the *blaP* gene of *B. licheniformis* flanked by translational stop codons, and in pKTH3262 a 4.6 kb *Eco*RV fragment of phage lambda. SDS-PAGE analysis of whole cell proteins of *E. coli* carrying these plasmids showed no full-size PrsA protein expressed by either plasmid, and a putative truncated PrsA of the expected size (14 kDa) expressed by pKTH3261 (data not shown). As a control, we constructed pKTH3253 in which the 5.3 kbp fragment was truncated for 1.9 kbp downstream of *prsA*, leaving this gene intact. In *B. subtilis* (IH6624 carrying pKTH10) the two plasmids with an insertion in *prsA* did not enhance the secretion of alpha amylase, while pKTH3253 did.

Enhanced secretion obtained by overproduction of *prsA* is of obvious advantage for large scale industrial production of exoenzymes. In such applications it is sometimes desirable to avoid the use of potentially unstable multicopy plasmids. One strategy is to insert one or few copies of the structural gene of the exoenzyme in the chromosome, combined with

altering its regulatory elements to increase expression. We therefore tested the effect of PrsA overproduction on the secretion of alpha amylase in such a system, where one copy of *amyE* was inserted in the chromosome fused to the target sequence of the regulatory protein DegQ in a strain overexpressing DegQ (A. Palva, personal communication). Also in this strain the high level of alpha-amylase secretion was enhanced about three fold by increasing the amount of PrsA protein (Table 1, strains BRB764 and IH67703). This indicates that the enhancement of secretion is achieved when the starting level of expression of exoprotein is high, regardless of the way the increased expression of the target gene has been obtained.

Turning now to the presence of PrsA in gram-positive bacteria other than *Bacillus subtilis*, we have confirmed the presence of PrsA or PrsA homologues in other species, *e.g., Bacillus amyloliquefaciens* and *Bacillus licheniformis*. The amount of PrsA protein in *Bacillus amyloliquefaciens* is similar to the amount in *Bacillus subtilis* cells, while the amount of PrsA protein in *Bacillus licheniformis* cells appears to be less. In addition, the components of the secretion machinery in these gram-positive bacillar strains are similar to that of *Bacillus subtilis*. *Bacillus amyloliquefaciens* and *Bacillus licheniformis* are two of the most widely used species of *Bacillus* in large scale industrial processes for the production of secreted proteases and amylases. The method of the invention using overproduction of PrsA protein to increase secretion of homologous and heterologous exoproteins of interest is especially useful in these strains.

As part of our invention we also teach how PrsA protein and/or the *prsA* gene can be identified in other gram-positive bacteria, and that functional homologues of the PrsA protein from *Bacillus subtilis* exist and can be used in the method and system of the invention. Functional homologues of the PrsA protein from *Bacillus subtilis* can be identified in other gram-positive species by using anti-PrsA antibody of high titer. Alternatively, the *prsA* gene or homologous *prsA*-like genes which encode functional homologues of the PrsA protein from *Bacillus subtilis* can be identified by Southern blotting using probe(s) from the *prsA* gene, or a *prsA*-gene

fragment. If the PrsA-like protein is found to exist, but there is insufficient homology to the *prsA* gene or PrsA protein to be detected unequivocally with antibodies specific for the PrsA protein, or DNA probes containing sequences homologous to the *prsA* gene, the homologous gene can be located and cloned, so unequivocal identification can be made.

However, in most cases homologous proteins and genes can be found using antibodies specific for the PrsA protein, or DNA probes containing sequences homologous to the *prsA* gene. For immunological identification, immunoblotting (Western blotting) can be used to detect the PrsA protein with anti-PrsA antibody of high titer. The antiserum is produced by immunizing an appropriate animal (*e.g.*, rabbit) with PrsA protein of *B. subtilis*, or preferably with a PrsA protein homologue of another species more closely related to the species of interest than *B. subtilis*. To identify the PrsA, the bacterium of interest can be grown on a number growth media, but preferably, the bacterium is grown on a medium where there is minimal induction of proteases. Bacterial cells are collected, again preferably at a growth phase with minimal amount of proteases present, and broken with a method appropriate for the species (usually a combination of enzymatic treatment and mechanical disrupture with sonication, French pressure cell, or shearing with glass beads). Samples of various sizes of broken cells and particulate fraction of the disrupted whole cells, prepared with ultracentrifugation, are electrophoresed in SDS-PAGE with standard methods, proteins are transferred to membrane filters and detected with anti-PrsA antiserum and the labeled second antibody. (Smaller amounts of PrsA protein can be detected if the particulate fraction is prepared). In all these steps standard methods and commercial reagents can be used.

To identify the *prsA* gene, or a *prsA*-like gene that encodes a functional homologue of PrsA in a species of interest, Southern blotting can be used. In this method, appropriate DNA probes from the *prsA* gene are hybridized to/with appropriately fragmented and electrophoresed chromosomal DNA of the species of interest according to the standard method of Southern hybridization. The hybridization probe may be any

fragment of DNA containing the *prsA* gene of *B. subtilis*, or a fragment of this gene, or a DNA fragment containing the *prsA* gene homologue of another species or a fragment of that gene. Once identified, the *prsA* gene homologue can be sequenced to further confirm its identity.

The teaching of the present invention includes not only overexpression of the PrsA protein of *Bacillus subtilis*, but also overexpression of a functional homologue of the PrsA protein from *Bacillus subtilis* in a gram-positive species of interest. According to the teaching of the invention, either the *prsA* gene of *B. subtilis* or the *prsA* gene homologue from another species, including the host species, is introduced into the host species. The *prsA* gene or its homologue is brought under the control of expression signals which are active in the species of interest in order to result in the high level (but not lethal) expression of the *prsA* gene. This can be accomplished in a variety of ways, including:

(1) The transfer of the plasmid pKTH277 to the species of interest. The transfer can take place with any method of transformation applicable to that species, like transformation, transduction, protoplast transformation, electroporation or conjugation. pKTH277 is maintained as multiple copy plasmid in many other gram-positive species other than *Bacillus*, and the expression signals of the *prsA* gene in that plasmid are active in many gram-positive species.

(2) Inserting the 5.3 kb *Sac*I fragment of pKTH277 into any other plasmid compatible with the species of interest and maintaining it at suitable copy number for high, but not lethal level of expression of *prsA*, relative to the activity of the *prsA* gene of *B. subtilis* in that species. The plasmid is then transferred to that species using any method of transformation applicable to that species. Alternatively, the fragment of DNA inserted into the plasmid can contain a *prsA* gene homologue of another species with its expression signals.

(3) Inserting the DNA fragment of pKTH277 encoding the signal sequence and the mature part of the PrsA protein to an expression vector suitable for the species of interest, under the control of expression signals in

that plasmid to achieve high level expression of *prsA*. As above, the appropriate fragment may derive from a *prsA* gene homologue of another species.

(4) The DNA constructions of paragraphs (2) and (3) above can be inserted into the chromosome of the species of interest instead of a plasmid. In that case, expression signals have to be chosen which are active enough to ensure high level expression of PrsA although there is only one copy of the gene per the genome.

As inventors who are also basic scientists, by design and of necessity much of our work is done under laboratory or simulated industrial conditions. However, with the help of industrial collaborators, it has been shown that the method and system of our invention work very well with commercially useful bacterial stains, under industrial fermentation conditions.

Methods and materials used in our studies, and examples of our invention are included below to further aid those skilled in the art in practicing the method and system of our invention.

Materials and Methods

Bacterial strains and plasmids

prs mutants of *Bacillus subtilis* Marburg 168 and their parent strains are shown in Table 1. Listed are also *B. subtilis* and *E. coli* strains overexpressing PrsA protein and *B. subtilis* strains with enhanced secretion of exoenzymes due to increased cellular amount of PrsA protein and their appropriate control strain. *E. coli* strains used as cloning hosts with plasmid vectors were HB101, TG1 and DHScc (Sambrook J., *et al.*, (1989) *Molecular cloning. A laboratory manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York) and with lambda, Kw25 1 (Promega, Madison, Wisconsin).

pHP13 (Haima, P., *et al.*, (1987) *Mol. Gen. Genet.*, 209:335-342, pJH101 (Ferrari, F.A., *et al.*, (1983) *J. Bacteriol.*, 154:1513-1515), pGEM3zf(+) (Promega) and pDR540 (Pharmacia, Upsala, Sweden) were used as cloning vectors for *prsA* gene and its fragments.

Properties of these plasmid vectors and constructed derivative plasmids carrying the *prsA* gene with a 5.3 kb (pKTH277 and pKTH268) or 3.4 kb (pKTH3253) insert are shown in Table 2. The *prsA* gene in pKTH3253 was disrupted by inserting a fragment either from *B. licheniformis blaP* gene (0.5 kb) or bacteriophage lambda genome (4.6 kb) in the unique *Eco*RV site in the ORF of *prsA* (the resulted plasmids were pKTH3261 and pKTH3262). pKTH10 (Palva, I. (1982a) *Gene,* 19:81-87; Palva, I., *et al.,* (1982b) *Proc. Natl. Acad. Sci. USA,* 79:5582-5586) and pMJ57 (Hastrup, S. and Jacobs, M.F. (1990) In Zukowski, M.M., *et al.,* (eds.), *Lethal phenotype conferred by xylose-induced overproduction of apr-lacZ fusion protein,* vol. 3. Academic Press, Inc., San Diego, California, pp. 33-41) are multicopy plasmids producing large amounts of *B. amyloliquefaciens* alpha-amylase (AmyE) and *B. licheniformis* subtilisin (SubC) secreted into the external medium, respectively. pKTH1582 has been constructed cloning the *amyL* gene from *B. licheniformis* on a secretion vector system of *B. subtilis* (BRB360 in Sibakov, M. (1986) *Eur. J. Biochem., 1sS,* 577-581; Palva, I. (1982a) *Gene,* 19:81-87; Palva, I., *et al.,* (1982b) *Proc. Natl. Acad. Sci. USA,* 79:5582-5586).

Growth media and culture conditions

Bacteria were grown in modified L-broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) with shaking at 37 degrees C, or on L-plates containing 1.5% agar (Difco, Detroit, Michigan) with appropriate antibiotics at +37 degrees C (Kontinen, V.P., *et al.,* (1991) *Mol. Microbiol.* 5:1273-1283); plates were modified for alpha amylase (5% starch and 2.5% agar) and subtilisin overexpressing strains (0.2% xylose and 1% milk powder). L-broth was supplemented with 2% soluble starch (Merck, Darmstadt, Germany) or used as 2-fold concentrated medium for production of exoenzymes. Strains producing subtilisin were grown on L-plates containing 1% milk powder. The production of exoenzymes was studied in two-fold concentrated L-broth with vigorous shaking. The growth was indicated by turbidity of the culture measured with KlettSummerson colorimeter (Klett Manufacturing Co., Inc. N.Y.) using a no. 66 filter.

Enzyme assays

Alpha amylase was assayed with Phadebas tablets (Pharmacia) as described in Kontinen, V.P. and Sarvas, M., (1988) *J. Gen. Microbiol.*, 134:2333-2344. For the plate assay bacteria were streaked on L-plate containing 5% of starch, and the halo around colonies was measured after incubation of the plates at 4 degrees C. Typically, there was no zone around wild-type colonies producing endogenous alpha amylase, while that of strains carrying pKTH10 was more than 2 mm depending on the age of plates. Subtilisin of *B. licheniformis* and chromosomally-encoded proteases were assayed in 1 ml of 0.1 M Tris-0.0 1 M CaCl2 (pH 8.0) with a chromogenic peptide substrate succinyl-AlaAla-Pro-Phe-p-nitroanilide (Del Mar, E.G., *et al.*, (1979) *Anal. Biochem.*, 99:316-320). The rate of hydrolysis was measured on a Hewlett Packard diode array spectrophotometer at 410 nm. To determine the specific activities of alpha-amylases and subtilisin their amount in a sample of culture supernatant was estimated either as in ((Kontinen, V.P. and Sarvas, M., (1988) *J. Gen. Microbiol.*, 134:2333-2344) or with SDS-PAGE stained with Coomassie Blue. The enzyme amounts were expressed as micrograms/ml.

Table 1. Bacterial strains

Strain reference	Relevant genotype and properties	Parent strain, source or
B.subtilis		
IH6064	*metB5 sacA321*	(Sibakov et al., 1983)
IH6090	*his metBS sac321*	IH6064
IH6157	IH6090 (pKTH10)	IH6090
IH6160	IH6064 (pKTH10)	IH6064
IH6480	*prs-3 metB5 sacA321* (pKTH10)	IH6157
IH8482	*prs-29 metB5 sacA321* (pKTH10)	IH6157
IH6483	*prs-33 metB5 sacA321* (pKTH10)	IH6 157

IH6484	*prs-40 metB5 sacA321* (pKTH10)	IH6157
IH6485	*prs-3 metB5 sacA321*	IH6480
IH6487	*prs40 metB5 sacA321*	IH6484
IH6489	*prs-ll metB5 sacA321* (pKTH10)	IH6160
IH649I	*prs-13 metBS sacA321* (pKTH10)	IH6160
IH6494	*prs-33 metB5 sacA321*	IH6483
IH6497	*prs-26 metB5 sacA321* (pKTH10)	IH6157
IH6498	*prs-13 metB5 sacA321*	IH6491
IH6501	*prs-26 metB5 sacA321*	IH6497
IH6504	*prs-ll metB5 sacA321*	IH6489
IH6513	QB917 (pKTH10)	QB917
IH6521	*prs-13 hisAI trpC2* (pKTH10)	IH6513
IH6523	*prs-ll metB5 sacA321* (pKTH10)	IH6513
IH6622	IH6624 (pKTH277)	IH6624
IH6623	IH6624 (pHP13)	IH6624
IH6624	PSLI (pKTH10)	PSLI
IH6654	*prs-29 metB5 sacA321*	IH6482
IH6752	PSLI (pMJ57)	PSLI
IH6755	PSLI (pHP13)	PSLI
IH6757	PSLI (pKTH1582)	PSLI
IH6759	IH6755 (pHP13)	IH6755
IH6760	IH6757 (pKTH277)	IH6757
IH6770	BRB764 (pKTH277)	BRB764
IH6774	IH6160 (pKTH277)	IH6160
IH6788	IH6752 (pKTH3229)	IH6752
IH6789	IH6752 (pKTH3230)	IH6752
PSLI	*arg(GH)15, leuA8, rm-, recE4, stp, thrA*	IA510 in BGSC
QB917	*hisAI thr-5 trpC2*	IAI0 in BGSC
BRB764	::Φ('P_{subE}'-*amyE*) (pKTH1743)	A. Palva, University of Helsinki

B.subtilis		
IH6559	*prsA29, recE4, trpC2* (::pKTH1601)	Kontinen *et al*, 1991
IH6799	IH6064 (::pKTH3200)	IH6064
IH6811	IH6624 (pKTH3253)	IH6624
IH6812	IH6624 (pKTH3261)	IH6624
IH6813	IH6624 (pKTH3262)	IH6624
E. coli		
EH1568	HBIOI (pKTH268)	HBIOI
EH1581	DH5α (pKTH3101)	DH5α
EH1631	TGI (pKTH3180)	TGI
EH1639	TGI (pGEM3zf(+))	TGI
EH 1640	TGI (pDR540)	TGI
EH1674	TGI (pKTH277)	TGI
EH1675	TGI (pKTH3253)	TGI
EH1678	TGI (pKTH3261)	TG1
EH 1679	TGI (pKTH3262)	TGI

1) pKTH1743 is a dreivative of pUB110 carrying a 0.3 kbp insert with an ORF for *B. subtilis decQ* gene

2) The Bacillus Genetic Stock Center, The Ohio State University, Ohio

Table 2. Plasmids

Plasmid number	Cloned genes and resistance markers	Derivative of (Source and/or references)
pKTH10	*amyE neo*	pUB110 (Palva, 1982; Gryczan et al., 1978)
pKTH268	*prsA bla*	pGEM3zf(+) (Promega;

			Sambrook et al., 1989)
	pKTH277	*prsA cat ermC*	pHP13 (Haima et al., 1987)
	pKTH1582	*amyL neo*	pUB110 (I. Palva; Sibakov, 1986)
5	pKTH1743	*degQ neo*	pUB110 (A. Palva)
	pKTH1786	*bla cat neo*	pAMβl (M. Simonen; Leblanc and Lee, 1984)
	pKTH3101	*'prsA bla*	pKTH268
	pKTH3180	O/P_{tac}-*'prsA bla*	pDR540 (Pharmacia)
10	pKTH3229	*ermC*	pHP13
	pKTH3230	*prsA ermC*	pKTH3229
	pKTH3253	*prsA cat ermC*	pHP13
	pKTH3261	0.5 kb insert in *prsA cat ermC*	pKTH3253
15	pKTH3262	4.5 kb insert in *prsA cat ermC*	pKTH3253
	pJH101	*bla cat tet*	(Ferrari et al., 1983)
	pMJ57	O/P_{xyn}-*'subC cat*	pUB110 (Hastrup and Jacobs, 1990

20

Examples

Example 1

25 Enhancement of alpha amylase secretion in *B. subtilis* when there is an increased amount of PrsA protein

The following table (Table 3) illustrates enhancement of α-amylase secretion in *Bacillus subtilis* under various conditions when the host gram-positive bacteria also overexpresses PrsA protein.

30

Table 3. Enhancement of α-amylase secretion in *B. subtilis* by overexpression of PrsA protein

Strain No.	α-amylase expressed from a gene in	The plasmid PrsA was expressed from		α-amylase secreted µg/ml[c]
		Plasmid No.	prsA gene in the plasmid	
IH6160	Multicopy plasmid[a]	---		1000
IH6774		pKTH277	Intact	3100
BRB764	Chromosome[b]	---		630
IH6770		pKTH277	Intact	2000
IH6811	Multicopy plasmid[a]	pKTH3253	Intact	3700
IH6812		pKTH3261	Disrupted	1300
IH6813		pKTH3262	Disrupted	1400

a) pKTH10 with *amyE* of *B. amyloliquefaciens*

b) One copy of *amyE* under a promoter of increased activity

c) The α-amylase activity of culture supernatant was assayed in the late stationary phase of growth

Example 2

Enhancement of alpha amylase secretion in *B. amyloliquefaciens* when there is hyperexpression of PrsA protein

This example demonstrates the effect of overproduction of PrsA protein of *B. subtilis* in *B. amyloliquefaciens* when the host gram-positive bacteria also hyperexpresses PrsA protein.

ALK02732 is a derivative of ALK02100 and contains the multicopy plasmid pKTHIO encoding the α-amylase of *B. amyloliquefaciens*. The strain thus contains tens of copies of α-amylase gene, and secretes about 20 fold more α-amylase than the wild type strain ALK02100 (Vehmaanperä *et al., J. Biotechnol.* 1991,19,221-240). Immunoblotting of cells of ALK02732 with anti PrsA antiserum showed that the amount of PrsA protein was similarly small in ALK02732 as in ALK02100, which is referred to below. Thus PrsA is rate limiting for protein secretion in ALK02732.

The plasmid pKTH277 was transferred with electroporation to the *B. amyloliquefaciens* strain ALK02732 to make ALK02732(pKTH277). The amount of PrsA in ALK02732(pKTH277) was many folds higher than in ALK02732, as determined with immunoblotting. This is in good agreement with the similar increase of PrsA proteins in *B. subtilis* strains transformed with PKTH277.

The amount of α-amylase secreted to the growth medium (Luria broth of double strength with 2% of soluble starch) by ALK02732 and ALK02732(pKTH277) were determined during logarithmic and early stationary phases of growth (shake flask cultures). The results are shown in Tables 4-1 (Exp. 1) and 4-2 (Exp. 2). It can be seen that in two separate experiments the amount of a-amylase in the culture medium of ALK02732(pKTH277) was 1.5 to 2.5 fold the amount in the growth medium of ALK02732.

Tables 4-1 and 4-2. The Effect of pKTH277 on a-amylase secretion of *B. amyloliquefaciens* (ALK02732).

Table 4-1, Experiment 1.

TIME(h)	ALK02732		ALK02732 (pKTH277)	
	GROWTH*	α-AMYLASE (mg/l)	GROWTH*	α-AMYLASE (mg/l)
3	106	1,1	100	1,1
4	241	8,8	240	8,8
5	426	47	430	72
6	520	160	525	224
7	576	480	580	650
8	625	800	630	1050

Table 4-2, Experiment 2.

TIME(h)	ALK02732 GROWTH*	ALK02732 α-AMYLASE (mg/l)	ALK02732 (pKTH277) GROWTH*	ALK02732 (pKTH277) α-AMYLASE (mg/l)
3	100	0,9	112	1,5
4	250	6	272	9
4,5	350	24	363	38
5	425	50	437	80
6,5	545	190	577	450
8	585	320	637	1300
12			702	1600
12,5	700	900		

* The density of the culture as determined with the Klett-Summerson colorimeter using filter no. 66, indicated with Klett units.

Materials and Methods Used in this Example

Bacterial strains and plasmids: *B. amyloliquefaciens* strain ALKO2732 (described by Vehmaanperä et al., 1991) was used in transformation and growth experiments. ALKO2732 (pKTH277) was made by transforming plasmid pKTH277 into ALK02732 (this study) and used in growth experiments. Plasmid pKTH277 carrying prsA gene is described by Kontinen et al. (1991).

Transformation of pKTH277 into ALK02732 by electroporation: Plasmid pKTH277 was isolated using the alkaline lysis method and methylated with BamHI methylase (New England Biolabs) according to manufacturers instructions. About 0.5 µg of methylated plasmid DNA was

used for electroporation. Electroporation was done as described by Vehmaanperä (1989). Cells were pulsed in 0.2cm sample cuvettes (Bio-Rad Laboratories) with Gene Pulser™ apparatus (Bio-Rad Laboratories) set at 1.5 kV, 25µF and 400Ω. Transformants were screened for chloramphenicol resistance on Luria-Kanamycin(10µg/ml)-Chloramphenicol(5µg/ml) plates. (Kanamycin was also on the plates to avoid loss of pKTH10, since ALK02732 contains pKTHIO, conferring kanamycin resistance).

Growth experiments and sample collection: First ALK02732 and ALK02732 (pKTH277) were grown over night on Luria plates. From the plates bacteria was added to 10 ml Luria and they were grown to logarithmic phase (Klett 100). Then 1ml Glycerol (1/10 of cultivation volume) was added and cell suspension was frozen and stored in -70C. Growth experiments were started by diluting Klett 100 cells 1:100 or 1:200 in 2x Luria + 2% starch. The Luria used in growth experiments contained no salt. Cultivation volume was 20 ml and growth was in bottles in 37C with vigorous shaking. For both strains Kanamycin (10µg/ml) and for ALK02732 (pKTH277) also Chloramphenicol (5µg/ml) was supplemented to growth medium. The growth was indicated by measurements with Klett-Summerson colorimeter (Klett Manufacturing Co., Inc. N.Y.) using a number 66 filter. 0.5 ml samples were taken during growth, samples were centrifugated and culture supernatants were stored in -20C for α-amylase assays.

α-amylase assays: α-amylases in culture supernatants were determined using Phadebas tablets (Pharmacia). Samples were incubated for 1h at 37C in 1ml buffer (50mM MES pH 6.8, 50mM NaCl, 100µM $CaCl_2$ containing 1/4 dispersed phadebas tablet, after which 50µl 5M NaOH was added to stop the reaction. After filtration through Whatman no.1 filter paper, absorbency of the filtrate was measured using 616-624nm as an analytical wave length range and 800-804nm as a reference wave length range. Commercially available α-amylase of *B. amyloliquefaciens* (Sigma) was used as a standard and results were expressed as mg enzyme per l.

References: Kontinen V., Saris P. and Sarvas M. (1991): A gene (prsA) of *Bacillus subtilis* involved in a novel, late stage of protein export. *Mol. Microbiol.* 5:1273-1283. Vehmaanperä, J. (1989): Transformation of *Bacillus Amyloliquefaciens* by electroporation. *FEMS Microbiol. Lett.* 61:165-170. Vehmaanperä J., Steinborn G. and Hofemeister J.(1991): Genetic manipulation of Bacillus amyloliquefaciens. *J. Biotechnol.*19:221-240.

Example 3

Enhancement of secretion of subtilis from *B. lentus* when there is hyperexpression of PrsA protein

This example demonstrates the method and system of the invention in enhancing secretion of overexpressed exoproteins in *B. lentus* when the host gram-positive bacteria also hyperexpresses PrsA protein.

The effect of hyperexpression of prsA has been tested with respect to the secretion of subtilisin from *B. lentus*, commercially available as Experase™ and described in WO 89/06279 (in the name of Novo Nordisk A/S). The subtilisin is transcribed from the plasmid pPL1800 which is based on the expression vector pPL1759 (Hansen, C., Thesis, 1992, The Technical University of Denmark) with a pUB110 origin and the promoter and signal peptide from the alpha-amylase of *B. licheniformis* (amyL). The plasmid pSX94 is described in WO 89/06279. The *B. subtilis* strain SHa273 used for production is a protease weak derivative of DN1885 (Jorgensen, P.L. *et al.*, (1991) *FEMS Microbiol. Lett.*, 77:271-276), in which two additional proteases apr and npr have been inactivated. The secretion of the *B. lentus* subtilisin was measured from strains either with (MOL253) or without (MOL252) the prsA plasmid pKTH277, and growth was performed at 30°C in soya broth BPX supplemented with kanamycin and chloramphenicol.

Measurements of subtilisin levels from the two strains after five days show that the strain with the prsA plasmid has a four fold higher secretion of the *B. lentus* subtilisin (160 micrograms/ml) compared to the strain without this plasmid (40 micrograms/ml).

The BPX medium used has the following composition:

BPX:	Potato starch	100	g/l
	Barley flour	50	g/l
	BAN 5000 SKB	0.1	g/l
	Sodium caseinate	10	g/l
	Soy Bean Meal	20	g/l
	Na_2HPO_4, 12 H_2O	9	g/l
	Pluronic	0.1	g/l

Strain List:

B. subtilis	Genotype and properties	Parent strain
DN 1885	amyE, amyR2	RUB200
PL1801	amyE, amyR2, apr-, npr-	DN1885
MOL252	PL1801 (pPL1800)	PL1801
MOL253	PL1801 (pPL1800, pKTH277)	PL1801

The RUB 200 strain is described by Yoneda *et al.*, 1979, Biochem Biophys. Res. Common. Vol. 91, 1556-64.

Example 4

The presence of PrsA protein in

Bacillus amyloliquefaciens and *Bacillus subtilis*

The presence of PrsA protein has been demonstrated in two strains of *B. amyloliquefaciens*, strain ALKO89 and strain ALKO2100. Strain ALKO89 is an industrial strain used for the production α-amylase. Strain ALK089 is an overproducer described in (Bailey, M.J. and Markkanen P.H. J. Appl. Chem. Biotechnol. 1975,25,73-79). Strain ALK02100 is a derivative of ATCC23843 (J. Vehmaanperä, *FEMS Microbiology Letters* 49(1988) 101-105).

The presence of PrsA protein has also been demonstrated in two strains of *Bacillus licheniformis*, strain 749/C (Pollock, M.R. (1965) Biochem. J. 94,666-6/5), and ATCC 14580.

The PrsA protein in these non *Bacillus subtilis* gram positive bacteria was identified using immunoblotting techniques. More specifically,

cells collected at late exponential phase of growth (to minimize the amount of proteases - PrsA protein is protease sensitive) were immunoblotted, treated with lysozyme shortly (to make cell wall leaky, but again avoiding long treatment to minimize proteolysis), and solubilized at 100°C with sample buffer containing 2% SDS. PrsA protein was detected with rabbit antiserum (KH1283) raised against PrsA protein of *B. subtilis* produced in *Escherichia coli* (thus there was minimal antigenic cross reaction with any bacillar protein except PrsA). The antiserum detects nanogram amounts of *B. subtilis* like PrsA in the immunoblots.

In addition, all four strains were found to contain a protein of the size of PrsA of *B. subtilis*, and specifically identified by the above antiserum. The intensity of the staining of the band was approximately similar in both *B. amyloliquefaciens* strains and similar to that of PrsA protein in wild type *B. subtilis*. Coomassie Blue staining of parallel SDS-PAGE of cellular proteins of *B. amyloliquefaciens* showed only a very weak band at the position of PrsA protein like in the case of *B. subtilis* and consistent with PrsA protein of *B. amyloliquefaciens* being a minor cellular protein as it is in *B. subtilis*. The intensity of staining of the PrsA protein of the two *B. licheniformis* strains was weaker than in B. subtilis, suggesting a somewhat smaller amount of PrsA protein. However, it cannot be excluded that the weaker staining is due to less efficient binding of the antiserum of PrsA protein of *B. licheniformis* than to that of *B. subtilis*. Table 5 is a summary of the roughly estimated amount of PrsA in the different strains.

Table 5. Approximate amounts of PrsA protein found in cells of Bacillus strains during early stationary growth phase (at a cell density of Klett 400). Estimates are based on Western blots and are preliminary.

Strain	PrsA in cells (arbitrary units)
B. amyloliquefaciens	
RH2078	4
RH2079	4[1)]

B. licheniformis		
	RH2080	1
	RH 305	1
B. subtilis		
	IH6064	5
	IH6774[2)]	1 00[1)]

1) Cells were collected from mid-logarithmic phase (at a density of Klett 100) of growth and the PrsA values estimated to correspond a density of Klett 400.

2) This is an overproducer of PrsA, due to the content of pKTH277.

Materials and Methods Used in this Example

Strains: *Bacillus amyloliquefaciens* RH2078 = ALKOB9 = VTTI97 = E18. This is an α-amylase overproducer. A gift from J.Vehmaanperä, ALKO. *Bacillus amyloliquefaciens* RH2079 = ALK02100, derived from ALK02099 (pE194/pC194). A gift from J. Vehmaanperä, ALKO. *Bacillus licheniformis* RH2080 = BRA5 = ATCCI4580. This is a producer of thermoresistent α-amylase. A gift from P.Saris, BI, H:ki. *Bacillus licheniformis* RH305 = 749/c. This is a penicillinase constitutive strain, originally derived from J.O.Lampen. *Bacillus subtilis* IH6074. This is *metB5 sacA321*.Ref. M.Sibakov et.al.1983. *Bacillus subtilis* IH6774. This is derived from IH6064, contains plasmids pKTH10 (carrying the α-amylase gene) and pKTH277(carrying the gene for coding PrsA).

Growth media: Luria-agar plates (L-plates); twice concentrated Luria-broth (2x L)

Purified PrsA protein: PrsA was purified from pKTH277 containing *B. subtilis* by M. Lauraeus.

Immune serum: *E. coli*-produced PrsA of *B. subtilis*, which was run in and cut out from a SDS-gel.

Chemicals: Phenylmethylsulfonylfluoride, Sigma P-7626. 100mM in ethanol at -20°C. EDTA, Titriplex®III p.a. Merck 8418. As a 0.5 M solution, pH 8. Lysozyme, Sigma L-6876. This was used as 1 mg/ml in the

following solution: 20mM potassium phosphate pH 7,15 mM $MgCl_2$, 20% sucrose. TCA 100% BCA† Protein Assay Reagent by Pierce.
SDS-PAGE and Western Blot equipment and chemicals according to BioRad. Blots were stained with 4-chloro-1-naphtol.

Culture conditions and sample preparation for gel electrophoresis: Bacteria were grown on L-plates overnight at 37°C. Colonies were picked with a glass rod into a preweighed Eppendorf tube, and weighed. Sample buffer was added to get either 10 or 100 mg cells(ww)/ml. Samples were heated 10 min at 100°C.

Bacteria were grown in 2xL broth with agitation at 37°C. To minimize the protease effect bacteria (from -20°C) were first grown to Klett 100 (corresponding to about 1-2 mg cells ww/ml, or about 10^9cells/ml). This was used as an inoculum at 10^{-2} dilution. 20 ml of bacteria were grown in Klett flasks and 4 ml samples were taken at Klett 100, at Klett 100+2h (Klett appr.400), and at Klett 100+4h (Klett appr.550).

Samples were immediately transferred into an ice bath, PMSF was added to 1 mM, and EDTA to 10 mM. Cells were separated from culture supernatant by centrifugation at 12 OOOxg 10 min., and treated with lysozyme, 15 min at 37°C in a 1/20 volume. An equal volume of sample buffer was added. Culture supernatant was precipitated in 10% TCA at 4°C and concentrated 20-fold in sample buffer.

The samples were run in 12% SDS-PA gels, stained with Coomassie Brilliant Blue R, or blotted onto PVDF filters according to BioRad.

PrsA was detected with the specific anti-PrsA rabbit antiserum KH 1283.

Example 5

Enhanced Secretion of Lipase from *Pseudomonas mendocina* in *Bacillus subtilis* that Overproduces PrsA Protein

Using the *prsA* gene in pKTH277, scientists at Genencor International, South San Francisco, CA, USA, have shown that when *Bacillus subtilis* overexpresses both PrsA protein and lipase (from *Pseudomonas*

mendocina, a gram-negative bacteria), the amount of lipase secreted into the medium is about 3.5 times greater than it is in controls that do not overexpress the *prsA* gene. The Genencor International scientists used industrial strains of *Bacillus*, and industrial fermentation conditions. (Data not shown).

Conclusion

Thus it can be seen that the present invention discloses a method and system for enhancing the production of industrially and medically important exoproteins in gram-positive bacteria. Without departing from the spirit and scope of this invention, one of ordinary skill can make various changes and modifications to the invention to adapt it to various usages and conditions. As such, these changes and modifications are properly, equitably, and intended to be, within the full range of equivalence of the following claims. Various features of the invention are also evident from the following claims.

Claims:

1. An expression system for enhancing secretion of exoproteins in gram-positive bacteria comprising a gram-positive bacteria capable of expressing greater than wild-type amounts of PrsA protein, or functional homologue(s) thereof, and capable of expressing greater than wild-type amounts of at least one exoprotein of interest.

2. An expression system according to claim 1 wherein said PrsA protein is homologous to said gram-positive bacteria.

3. An expression system according to claim 1 wherein said PrsA protein is heterologous to said gram-positive bacteria.

4. An expression system according to claim 1 wherein said PrsA protein is PrsA protein from a species of *Bacillus*.

5. An expression system according to claim 4 wherein said PrsA protein is PrsA protein from *Bacillus subtilis*, *Bacillus amyloliquefaciens* or *Bacillus licheniformis*.

6. An expression system according to claim 1 wherein said functional homologue of said PrsA protein is immunologically reactive with antibody raised against PrsA protein from *Bacillus subtilis*, *Bacillus amyloliquefaciens*, or *Bacillus licheniformis*, and when overexpressed, is capable of enhancing secretion of said exoprotein of interest from said gram-positive bacteria.

7. An expression system according to claim 1 wherein said PrsA protein, or functional homologue thereof, is present in said gram-positive cell in amounts that are from 2 to about 10 times greater than wild-type amounts.

8. An expression system according to claim 1 wherein said exoprotein of interest is a protease, a lipase, a cutinase, an amylase, a galactosidase, a pullulanase, a cellulase, a glucose isomerase, a protein disuphide isomerase, a CGT'ase (cyclodextrin gluconotransferase), a phytase, a glucose oxidase, a glucosyl transferase, laccase, bilirubin oxidase, a xylanase, an antigenic microbial or protozoan protein, a bacterial protein toxin, a microbial surface protein, a viral protein, a pharmaceutical.

9. An expression system according to claim 8 wherein said exoprotein of interest is a non native exoprotein that has been created by the addition of a signal sequence to the structural gene encoding said protein.

10. An expression system according to any of claims 1-9 wherein said gram-positive bacteria is a species of *Bacillus*.

11. An expression system according to Claim 10 wherein said *Bacillus* is *Bacillus subtilis, Bacillus licheniformis, Bacillus lentus, Bacillus brevis, Bacillus stearothermophilus, Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus coagulans, Bacillus circulans, Bacillus lautus* and *Bacillus thuringiensis*.

12. A gram-positive bacteria able to express greater than wild-type amounts of at least one exoprotein of interest further comprising pKTH277.

13. A gram-positive bacteria able to express greater than wild-type amounts of at least one exoprotein of interest and further comprising at least one of the following: at least two copies of the *prsA* gene from *Bacillus subtilis*, or a functional homologue thereof; the *prsA* gene from *Bacillus subtilis*, or a functional homologue thereof, operatively linked to strong regulatory sequences which result in overexpression of said *prsA* gene, or functional homologue thereof.

14. A gram-positive bacteria according to any of claims 12 or 13 wherein said gram-positive bacteria is a bacteria from the genus *Bacillus*.

15. A gram-positive bacteria according to Claim 14 wherein said *Bacillus* is *Bacillus subtilis, Bacillus licheniformis, Bacillus lentus, Bacillus brevis, Bacillus stearothermophilus, Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus coagulans, Bacillus circulans, Bacillus lautus* and *Bacillus thuringiensis*.

16. A DNA construct comprising the *prsA* gene from *Bacillus subtilis*, or a functional homologue thereof, under the control of expression signals which cause overexpression of said *prsA* gene, or functional homologue thereof.

17. A vector further comprising the *prsA* gene from *Bacillus subtilis*, or a functional homologue thereof, under the control of expression signals which cause overexpression of said *prsA* gene, or functional homologue thereof.

18. A method for enhancing secretion of an exoprotein of interest in a gram-positive bacteria comprising expressing greater than wild type amounts of PrsA protein from *Bacillus subtilis*, or a functional homologue thereof in said gram-positive bacteria, wherein said gram-positive bacteria is also capable of expressing greater than wild type amounts of said exoprotein.

19. A method according to claim 18 wherein said gram-positive bacteria is a bacteria from the genus *Bacillus*.

20. A method according to claim 19 wherein said *Bacillus* bacteria is *Bacillus subtilis, Bacillus licheniformis, Bacillus lentus, Bacillus brevis, Bacillus stearothermophilus, Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus coagulans, Bacillus circulans, Bacillus lautus* or *Bacillus thuringiensis*.

21. A method for creating an improved non-*Bacillus subtilis* gram- positive host organism useful for enhanced secretion of an exoprotein of interest that is overexpressed in said host organism, said method comprising (a) identifying a gene from said host organism that encodes a functional homologue of PrsA protein from *Bacillus subtilis*, (b) enhancing the expression of said gene identified in step (a) by at least one of the following: introducing into said host organism at least one additional copy of said gene; introducing into said host organism said gene operatively linked to expression sequences which result in overexpression of said gene.

22. A method for identifying a gene which encodes a functional homologue of PrsA from *Bacillus subtilis*, said method comprising identifying, by means of Southern blotting, DNA which hybridizes with DNA probe(s) from the *prsA* gene from *Bacillus subtilis*, and demonstrating that said gene encodes a protein which when overexpressed, is capable of enhancing the secretory capability of a gram-positive bacteria with respect to secretion of an exoprotein of interest.

23. A method for identifiying a gene which encodes a functional homologue of PrsA from *Bacillus subtilis*, said method comprising identifying protein that reacts with anti-PrsA antibod(ies) of high titer, and demonstrating that when said protein is present in greater than wild-type amounts in a gram-positive bacteria, said protein is capable of enhancing the secretory capability of said gram-positive bacteria with respect to secretion of an exoprotein of interest.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 94/00072

A. CLASSIFICATION OF SUBJECT MATTER

IPC : C12N 15/67, C12N 15/74, C12N 15/75
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC : C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, WPI, CLAIMS, US PATENT FULLTEST

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Dialog Information Servies, File 155, Medline, Dialog accession no. 07927824, Medline accession no. 92065824, Kontinen VP et al, "A gene (prsA) of Bacillus subtilis involved in a novel, late stage of protein export". Mol Microbiol (England) May 1991, 5 (5) p 1273-83 --	1-23
Y	EP, A1, 0227260 (BIOTECHNICA INTERNATIONAL, INC.), 1 July 1987 (01.07.87), page 13, line 16 - page 14, line 7, claim 13 --	1-23

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 June 1994	17 -06- 1994
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Jonny Brun Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 94/00072

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	Dialog Information Services, File 155, Medline, Dialog accession no. 08613751, Medline accession no. 93323751,Kontinen VP et al, "The PrsA lipoprotein is essential for protein secretion in Bacillus subtilis and sets a limit for high-level secretion". Mol Microbiol (ENGLAND) May 1993, 8 (4) p 727-37	1-23

--

INTERNATIONAL SEARCH REPORT

Information on patent family members

28/05/94

International application No.
PCT/FI 94/00072

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A1- 0227260	01/07/87	DE-A,T- 3688282	19/05/93
		JP-A- 62181778	10/08/87
		US-A- 5017477	21/05/91